A Putative 'Pre-Nervous' Endocannabinoid System in Early Echinoderm Development

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Abstract
Embryos and larvae of sea urchins *(Lytechinus variegatus, Strongylocentrotus droebachiensis, Strongylocentrotus purpuratus, Dendraster excentricus),* and starfish *(Pisaster ochraceus)* were investigated for the presence of a functional endocannabinoid system. Anandamide (arachidonoyl ethanolamide, AEA), was measured in early *L. variegatus* embryos by liquid chromatography/mass spectrometry. AEA showed a strong developmental dynamic, increasing more than 5-fold between the 8–16 cell and mid-blastula 2 stage. 'Perturb-and-rescue' experiments in different sea urchin species and starfish showed that AEA blocked transition of embryos from the blastula to the gastrula stage, but had no effect on cleavage divisions, even at high doses. The non-selective canna-binoid receptor agonist, CP55940, had similar effects, but unlike AEA, also blocked cleavage divisions. CB1 antagonists, AEA transport inhibitors, and the cation channel transient membrane potential receptor V1 (TrpV1) agonist, arachidonoyl vanillic acid (arvanil), as well as arachidonoyl sero-tonin and dopamine (AA-5-HT, AA-DA) acted as rescue substances, partially or totally preventing abnormal embryonic phenotypes elicited by AEA or CP55940. Radioligand binding of [^{3}H]CP55940 to membrane preparations from embryos/larvae failed to show significant binding, consistent with the lack of CB receptor orthologs in the sea urchin genome. However, when binding was conducted on whole cell lysates, a small amount of [^{3}H]CP55940 binding was observed at the pluteus stage that was displaced by the CB2 antagonist, SR144528. Since AEA is known to bind with high affinity to TrpV1 and to certain G-protein-coupled receptors (GPCRs), the ability of arvanil, AA-5-HT and AA-DA to rescue embryos from AEA teratogenesis suggests that in sea urchins AEA and other endocannabinoids may utilize both Trp and GPCR orthologs. This possibility was explored using bioinformatic and phylogenetic tools to identify candidate or-

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thologs in the *S. purpuratus* sea urchin genome. Candidate TrpA1 and TrpV1 orthologs were identified. The TrpA1 ortholog fell within a monophyletic clade, including both vertebrate and invertebrate orthologs, whereas the TrpV1 orthologs fell within two distinct TrpV-like invertebrate clades. One of the sea urchin TrpV orthologs was more closely related to the vertebrate epithelial calcium channels (TrpV5-6 family) than to the vertebrate TrpV1-4 family, as determined using profile-hidden Markov model (HMM) searches. Candidate dopamine and adrenergic GPCR orthologs were identified in the sea urchin genome, but no cannabinoid GPCRs were found, consistent with earlier studies. Candidate dopamine D_1, D_2 or α_1-adrenergic receptor orthologs were identified as potential progenitors to the vertebrate cannabinoid receptors using HMM searches, depending on whether the multiple sequence alignment of CB receptor sequences consisted only of urochordate and cephalochordate sequences or also included vertebrate sequences.

Introduction

Accumulated evidence supports the existence of functional neurotransmitter systems (e.g., acetylcholine, serotonin, dopamine) that regulate key aspects of early ('prenervous') embryonic development in vertebrate and invertebrate species, including sea urchins [Buznikov, 1967; Gustafson and Toneby, 1970; Gutafson and Toneby, 1971; Gustafson et al., 1972; Kirby and Gilmore, 1972; Toneby, 1974; Lenicque et al., 1977; Palen et al., 1979; Wallace, 1982; Buznikov, 1990; Buznikov et al., 1996; Colas et al., 1999a; Colas et al., 1999b; Levin and Mercola, 1999; Buznikov et al., 2001a; Buznikov et al., 2005; Levin et al., 2006]. Sea urchins are the most studied invertebrate model for investigation of neurotransmitter functions during early embryonic stages [Buznikov, 1967; Gustafson and Toneby, 1970; Gustafson et al., 1972; Renaud et al., 1983; Buznikov, 1990; Buznikov et al., 2001a; Qiao et al., 2003; Anitole-Misleh and Brown, 2004; Katow et al., 2004].

Anandamide (AEA) was the first endogenous agonist for cannabinoid receptors to be discovered [Devane et al., 1992]. It mimics many of the pharmacological properties of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive substance in *Cannabis* [Freund et al., 2003]. AEA is also an agonist for vanilloid (TrpV1 and ANKTM1) receptors, and directly inhibits currents generated by L-type Ca^{2+} and TSK-1K^+ channels [Di Marzo et al., 2001; Wang et al., 2006c]. The synthetic AEA analog, R-methanandamide (AM-356) directly blocks acetylcholine-me-

diated responses in *Xenopus* oocytes transfected with α_7-nicotinic receptors (IC_{50} approx. 168 nM), while THC and synthetic CB receptor agonists (CP55940 and WIN552122) do not affect α_7-nicotinic receptor functions [Oz et al., 2004]. AEA is released from membrane phospholipid precursors, *N*-acyl-phosphatidylthanolamines (NAPEs), by sequential actions of membrane-bound Ca^{2+}-dependent transacylase and phospholipase D (NAPE-PLD) when neurons and other cells are stimulated by depolarizing agents, neurotransmitters, and hormones [Freund et al., 2003; Okamoto et al., 2004]. High levels of NAPE-PLD are found in brain and testis [Okamoto et al., 2007]. Released AEA is quickly taken up from the extracellular space by a transporter and hydrolyzed by a membrane-bound fatty acid amide hydrolase (FAAH), indicative of roles in cell signaling [Freund et al., 2003; Maccarrone and Finazzi-Agro, 2004]. Retrograde AEA signaling modulates secretion of neurotransmitters at synapses in central and peripheral neurons [Alger, 2002].

The current study sought to provide evidence for an endogenous cannabinoid (endocannabinoid) system in embryos of sea urchins and other echinoderms, based on results of an earlier drug screen [Buznikov et al., 2002], and evidence for a putative CB1-like signaling system in sea urchin sperm, which is involved in fertilization and prevention of polyspermy [Chang et al., 1993; Schuel et al., 1994; Berdyshev, 1999; Schuel and Burkman, 2005]. The possibility that fertilized sea urchin eggs and embryos might also synthesize AEA was suggested by the presence of NAPE-PLD and FAAH in sea urchin eggs [Bisogno et al., 1997]. AEA is synthesized by the uterus and appropriate regulation of uterine levels by NAPE-PLD and FAAH is critical for all stages of prenatal development [Fride and Shohami, 2002; Fride, 2008]. Preimplantation mouse embryos use their own PLD and FAAH to produce an endogenous AEA tone that regulates development to the mature blastocyst [Schuel, 2006; Wang et al., 2006c; Wang et al., 2008]. During early pregnancy, optimally low levels of AEA are required for preparation of the blastocyst, its passage through the oviduct, and implantation at appropriate sites [Paria et al., 2002; Wang et al., 2006a; Fride, 2008]. Inappropriately high levels of AEA disrupt embryonic development by inhibiting cell proliferation, promoting apoptosis [Turco et al., 2008] and preventing implantation [Wang et al., 2006a]. AEA regulates blastocyst development and implantation by activating CB1 receptors [Paria et al., 1995; Paria et al., 1998; Paria and Dey, 2000; Paria et al., 2002], and plays various other roles in pre- and postnatal development [Fride, 2008; Patinkin et al., 2008] in mammals, as well as in

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Table 1. Teratogenic actions of cannabinoid/vanilloid receptor ligands on development of echinoderm embryos/larvae

Substance	Source	Pharmacological activity	Dose μM	Effects	Rescue compounds	Species
Anandamide AA-EG (AEA)	VVB[b] NIDA drug supply	type 1 and type 2 cannabinoid and vanilloid (TrpV1) receptor agonist	10–50	disturbs development from the late blastula stage	AM-251, AM-630, ACPA, AA-EG[b] (AEA), arvanil, AM-404, VDM-11, SR141716A, AA-5-HT	1 (35) 2 (10) 5 (6) 6 (2)
CP55940	Sigma Tocris NIDA drug supply	cannabinoid type 1 and type 2 cannabinoid receptor agonist	≥1–2	disturbs development from the first cleavage divisions	AA-EG[b] (AEA), AM-251, SR141716A, AA-5-HT[b], AA-Tyr[b]	1 (5) 2 (15) 3 (2) 4 (6) 5 (9)
WIN55212-2	Sigma	cannabinoid type 1 and type 2 cannabinoid receptor agonist	20–40	disturbs development after onset of gastrulation	AM-404, arvanil	1 (2)
AM-630	Tocris	type 2 cannabinoid type 2 receptor antagonist	≥1–2	disturbs development from the first cleavage divisions	AEA, AM-251, AA-EG[b] (AEA), ACPA, arvanil, AA-DA[b]	1 (11) 2 (2)
Capsazepine	Tocris	vanilloid (TrpV1) antagonist	≥2.5	disturbs development from the first cleavage divisions	not known; VR agonists did not protect	1 (4) 2 (9)
SB366791	Sigma	vanilloid (TrpV1) antagonist	≥1	disturbs development from the blastula stage	AM-251, AM-630, AM-404, arvanil, AA-5-HT[b], AA-DA[b], forskolin	2 (11)

AEA (AA-EG). Species: sea urchins: 1 = *Lytechinus variegatus;* 2 = *Strongylocentrotus droebachiensis;* 3 = *Strongylocentrotus purpuratus;* 4 = *Dendraster excentricus;* starfish = 5. *Pisaster ochraceus;* 6 = *Evasterias troschelii.*
Number of experiments are given in parentheses.
[a] From commercial sources.
[b] Synthesized in the laboratory of V.V. Bezuglov (Institute of Bioorganic Chemistry, Moscow, Russia).

frogs and fish, where it regulates hatching and motility of larvae [Migliarini et al., 2006; Migliarini and Carnevali, 2008; Migliarini and Carnevali, 2009]. Preliminary experiments on sea urchin embryos and larvae had shown that cannabinoid/vanilloid receptor ligands, like AEA and arachidonoyl ethylene glycol (AA-EG), prevent malformations caused by lipophilic acetylcholine receptor agonists [Buznikov et al., 2001b]. Therefore, we reasoned that an endogenous cannabinoid system might play a role in early echinoderm development, such as regulating cleavage divisions, cell differentiation, or morphogenetic cell movements during blastulation and gastrulation.

Materials and Methods

Animals
The majority of pharmacologic experiments were performed on embryos and larvae of sea urchins, *Lytechinus variegatus* and *Strongylocentrotus droebachiensis*. Other echinoderms (sea urchins, *Strongylocentrotus purpuratus* and *Dendraster excentricus*, and starfish, *Pisaster ochraceus*) were also used in some experiments. Proteomic studies were only performed on *L. variegatus* embryos/larvae. Adult specimens of *L. variegatus* were main-

tained in tanks with filtered, circulating artificial sea water (ASW), whereas other echinoderm species used at Friday Harbor Laboratories (University of Washington, Wash., USA) were maintained in tanks with running natural sea water.

Pharmacologic 'Perturb and Rescue'
Fertilization. Adult sea urchins were injected with 0.55 M KCl, and gametes harvested. Suspension of mature oocytes of starfish were transformed to eggs by treatment with 1 μM 1-methyladenine. In both cases, eggs were washed with ASW, fertilized, and embryos/larvae incubated in multiwell tissue culture plates (100–150 specimens/well) with drug or vehicle, as described previously [Buznikov et al., 2003].
Pharmacologic Treatment. All experiments were conducted at approximately 21°C, for *L. variegatus,* or at 10°C for other echinoderm species. Test substances were introduced after fertilization, the first cleavage divisions, mid-blastula 2, early or late gastrula stages, at a final concentration of 1–100 μM. Putative rescue substances were given together with corresponding teratogen, or 10–15 min earlier. Each substance was tested on embryos/larvae in multiple experiments (tables 1, 2), where embryos (2,000–3,000) from a single female were used in each experiment. Control specimens were incubated in ASW with the same volume of vehicle used to dissolve test chemicals (DMSO, ethanol, methanol or deionized/distilled water). Results (changes in cell and/or embryonic/larval phenotype) were documented by brightfield imaging with a Leitz Orthoplan microscope and Spot RT color digital

Table 2. Cannabinoid/vanilloid receptor ligands as rescue compounds

Substance	Source	Pharmacological activity	Dose μM	Protective action against	Species[a]
Anandamide (AEA)	VVB[b] NIDA drug supply	type 1 and type 2 cannabinoid, and vanilloid receptor agonist	10–20	AM-630, CP55940	1 (3) 5 (3)
Arachidonoyl-ethylene glycol AA-EG[a] (AEA)	VVB[b]	type 1 and type 2 cannabinoid receptor agonist	40	AA-EG[a] (AEA), CP55940, AM-630, chlorpyrifos, arachidonoylcholine, ritanserin	1 (13) 2 (13) 3 (2) 4 (3) 5 (5)
ACPA	VVB[b] Tocris	type 1 cannabinoid receptor agonist	40	AA-EG[a] (AEA), AM-630, chlorpyrifos	1 (4) 2 (4)
AM-251	Tocris	type 1 cannabinoid receptor antagonist	10–20	AA-EG[a] (AEA), CP55940, AM-630, SB366791	1 (6) 2 (24) 3 (2) 4 (6) 5 (3) 6 (2)
SR141716A	NIDA drug supply	type 1 cannabinoid receptor antagonist	10	AA-EG[a] (AEA), CP55940	1 (3)
AM-630	Tocris	type 2 cannabinoid receptor antagonist	≥1–2 and more	AA-EG[a] (AEA), SB366791	1 (11) 2 (2)
Arachidonoyl vanillic acid (arvanil)	VVB[b]	cannabinoid/vanilloid receptor agonist	40	AA-EG[a] (AEA), CP55940, WIN55212-2, SB366791, AM-630, ritanserin, cinanserin, chlorpyrifos, lipophilic derivatives of choline, retinoic acid	1 (33) 2 (44) 3 (2) 4 (6) 5 (22) 6 (2)
AM-404	Tocris	inhibitor of anandamide transport	10–50	AA-EG[a] (AEA), CP55940, WIN55212-2, SB366791	1 (22) 2 (1)
VDM-11	Tocris	inhibitor of anandamide transport	20–50	AA-EG[a] (AEA)	1 (3)
Arachidonoyl tyramine (AA-Tyr)	VVB[b]	analog of AM-404	40	CP55940, lipophilic derivatives of choline, ritanserin	1 (3) 5 (7)

Footnotes are the same as in table 1.

camera (Diagnostic Instruments). Imaging was performed when control embryos or larvae were at the mid-blastula, early-late gastrula or early prism stage. Malformation phenotypes were scored as present when 80–100% of the specimens exhibited a given phenotype at a particular drug concentration, which was completely absent in vehicle controls.

Pharmacologic characteristics of drugs used in these experiments, as determined in vertebrates, are listed in tables 1 and 2. Lipophilic derivatives of particular neurotransmitters [arachidonoyl serotonin (AA-5-HT), arachidonoyl dopamine (AA-DA), arachidonoyl choline (AA-Ch)] and arachidonoyl vanillic acid (arvanil), synthesized by V.V. Bezuglov and collaborators, were utilized, as well as 5-HT$_2$ receptor antagonists, ritanserin (Sigma) and cinanserin (Tocris), the cholinergic insecticide, chlorpyrifos

(ChemService, West Chester, Pa., USA), forskolin, a direct activator of adenylyl cyclase (Tocris), capsaicin, capsazapine and resinferatoxin (Tocris), and arvanil (NIDA drug supply).

Liquid Chromatography/Mass Spectrometry (LC/MS) of AEA
Methods for tissue extraction of AEA were based on those described by Cravatt et al. [2001], with modifications. A 2:1:1 solution of CHCl$_3$/MeOH/50 mM Tris pH 8 (8 ml/0.4 g tissue) was prepared for tissue extraction. For targeted LC measurements, deuterated AEA (AEA-d$_8$; 0.5 pmol, Cayman Chemicals) was included in the solution [Lam et al., 2008]. Embryos/larvae at the 2-cell, 8–16 cell, and mid-blastula 2 stages were harvested by gentle centrifugation (2,000 *g*), placed into CHCl$_3$/MeOH/50 mM Tris pH 8 solution, and homogenized using dounce tissue grind-

ers. Each sample was centrifuged at 2,000 g for 10 min at 4 °C in a glass vial. After centrifugation, the organic and aqueous layers were clearly distinguishable with a layer of insoluble material between them. The organic layer was carefully removed, and concentrated under a stream of nitrogen gas. Samples were dissolved in 300 µl MeOH prior to analysis by LC/MS.

Detection and quantification of AEA by LC/MS was performed as previously described [Di Marzo et al., 2000], with modifications. AEA was detected using a C18 reversed phase HPLC column (Zorbax C18, 2 × 50 mm, 1.8 µm particle size) operated with a linear gradient of 80–100% methanol containing 10 mM ammonium acetate over 6 min at a flow rate of 250 µl/min, interfaced to a TSQ Quantum (Thermo, San Jose, Calif., USA) triple quadruple mass analyzer. AEA and AEA-d_8 were detected in selective reaction monitoring mode, monitoring the transitions of m/z 348.4 to 105 and m/z 356.4 to 108.1 for AEA and AEA-d_8, respectively. Prior to analysis, the instrument was optimized using the compound optimization function of the Xcalibur software.

Immunocytochemistry

Primary Mesenchyme Cells (PMC). Primary antibody, 1D5, [Wessel and McClay, 1985] was used to label PMC in normal embryos/larvae and those treated with AEA or AEA + arvanil. Specimens were fixed in cold 2% paraformaldehyde in 60% ASW for 10 min at room temperature. Following fixation, they were washed in cold methanol, placed in small multiwell (Terasaki) plates, washed 3 × 10 min in PBS/2% normal goat serum (PBS-NGS) directly in the wells, and incubated with primary antibody 1D5 (1:250–1:500) or no primary antibody (control) overnight at 4°C. Specimens were washed 3 × 10 min in PBS-NGS and incubated with Cy2-labeled (green) secondary antibody (IgM, 1:500; Jackson Immuno Research Labs) for 1 h in the dark at room temperature. Following the secondary antibody, specimens were again washed in PBS-NGS, and run through an ascending glycerol series (30, 50, 70% in PBS, for 1 h each), mounted on glass slides, coverslipped, and imaged on a Zeiss LSM510 confocal microscope.

Receptor Binding

L. variegatus sea urchin larvae were collected at the pluteus stage by centrifugation (1,600 g, 30 s). The supernatant was discarded, the pellets were weighed, then frozen in a dry ice/isopropanol bath and stored at –80°C until use. Whole cell lysates were prepared by adding 2 mg/ml RIPA cell lysis buffer (Pierce, Rockford, Ill., USA) with 1% Triton and protease inhibitor cocktail (5 µl/mg protein, Chemicon) and homogenized at 4°C, then placed on an orbital shaker for 2 h at 4°C. Lysate was then centrifuged in a precooled centrifuge for 20 min at 125,000 g and protein was measured in the pellet using the Bio-Rad assay. Rat brain or spleen P2 membrane fractions were prepared by homogenization (10 strokes) in a buffer comprised of 320 mM sucrose, 2 mM Tris-EDTA, 5 mM MgCl$_2$ followed by two rounds of washing and centrifugation (5,200 g for 10 min at 4°C). The supernatants were combined and further centrifuged twice (40,000 g at 4°C for 25 min) and the pellet was resuspended in TME buffer (50 mM Tris-HCl, pH 7.4, 3 mM MgCl$_2$, 1 mM EDTA) for protein determination using the Bio-Rad assay.

Radioligand binding was performed as previously described [Devane et al., 1988; Francisco et al., 2002]. The non-selective cannabinoid receptor ligand, [^{3}H]CP55940 (specific activity: 0.66 mCi/ml; 2 µM, NIDA Drug Supply), was used to probe sea urchin whole cell lysates (100 µg protein) or P2 membrane fractions from rat brain (25 µg) or spleen (50 µg). The selective ligands SR141716 (5 µM, NIDA) and SR144528 (1 µM, NIDA) were used to define binding to CB1 or CB2 receptors, respectively. Whole cell lysates or P2 membranes were added to TME assay buffer containing 50 mM Tris-HCl, pH 7.4, 3 mM MgCl$_2$, 1 mM EDTA containing 0.2% fatty acid-free BSA. The final assay volume of 500 µl was incubated for 60 min at 30°C. The reaction was terminated by rapid filtration under vacuum (<25 in Hg) using Whatman GF/C filter strips. Filters were washed twice with 3-ml aliquots of assay buffer at 0–4°C, dried, dissolved in EcoScintH liquid scintillation cocktail (5 ml) and counted by liquid scintillation spectroscopy. Between-group comparisons were made by ANOVA followed by post-hoc tests (Tukey or Newman-Keuls) using GraphPad Prism 4.0.

Bioinformatics

Sequence Retrieval and Database Searches. Candidate echinoderm and ascidian orthologs of vertebrate G-protein-coupled receptor (GPCR) cannabinoid receptors and the cation channel transient membrane potential (Trp) receptors were identified by blast sequence similarity searches [Altschul et al., 1997] using human and mouse orthologs from the UniProt Knowledgebase (http://www.expasy.org) as query sequences. Queries were searched against the echinoderm *S. purpuratus* sea urchin genome, both the GLEAN3 gene (protein) predictions database (http://www.hgsc.bcm.tmc.edu/blast.hgsc) and the NCBI sea urchin reference sequences database, and two ascidian genomes, *Ciona intestinalis* (sea squirt) and *Ciona savignyi* (Pacific sea squirt) (www.ensembl.org). *Drosophila melanogaster* (fruitfly) and *Caenorhabditis elegans* (worm) TrpV and TrpA orthologs and GPCR receptors of the dopamine-related family from the UniProt Knowledgebase (http://www.expasy.org) were also used as query sequences to search the ascidian and echinoderm genomes. In addition, orthologs identified in the echinoderm family were used to search the ascidian genome, and vice versa. Echinoderm and ascidian homologs were retained for further analysis if they retrieved the original query protein as the top hit. The published *Branchiostoma floridae* CB sequence [Elphick, 2007] was also included in some analyses.

The gene predictions for the sea urchin TrpA1 ortholog from the NCBI refseq database, XP_782013.2, and the GLEAN3 gene prediction from the Baylor database, GLEAN3 15403, each contained an insertion that was not in the other sequence. Therefore, a consensus sequence that was a composite, containing both insertions, was used in this analysis, since both insertions were common to TrpA1 proteins from other species. The UniProt, NCBI refseq, GLEAN3 or Ensembl IDs for the sequences included in these analyses are contained within the protein name in the TrpV and GPCR multiple sequence alignments (online suppl. fig. S1, S2, www.karger.com/doi/10.1159/000235758).

HMMER v2.3.2 (http://hmmer.janelia.org/) was used to complete profile hidden Markov model (HMM) searches of the sea urchin genome database. The queries were a series of multiple sequence alignments containing different subsets of TrpV and GPCR cannabinoid receptor sequences. For the TrpV searches, the query alignments contained only sequences from vertebrates, whereas the GPCR query alignments contained cannabinoid receptors from the urochordates *C. intestinalis* and *C. savignyi*, and the cephalochordate *B. floridae* alone, or together with vertebrate
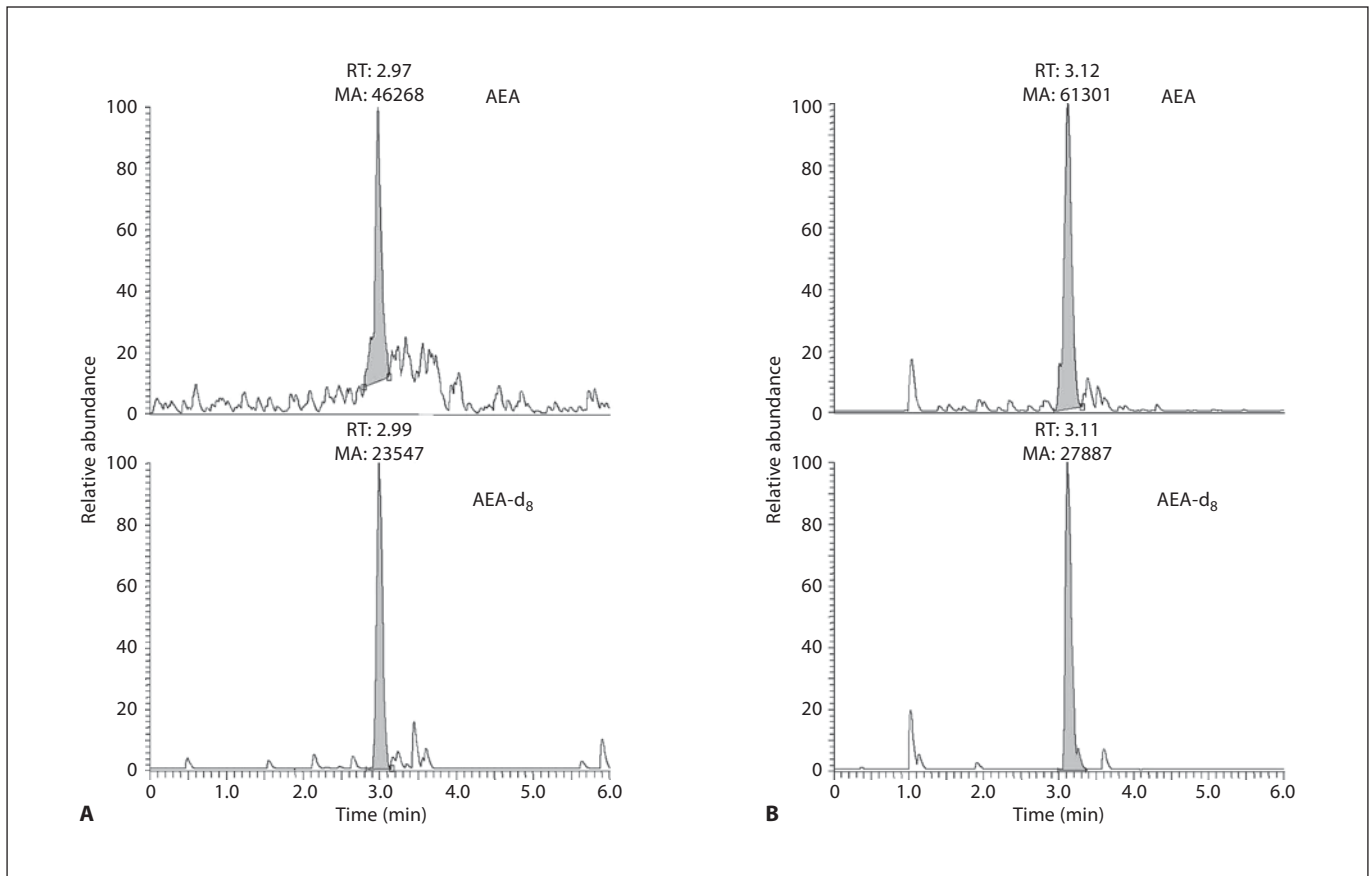
Fig. 1. Detection and measurement of AEA by LC/MS in *L. variegatus* embryos. Ion chromatograms of AEA and AEA-d$_8$ in **A** 8–16 cell stage and **B** mid-blastula 2 stage embryos. AEA was quantified using AEA-d$_8$ as internal standard, where 90 pmol was added to the sea urchin samples. The amounts of AEA measured at the 8–16 cell and mid-blastula stage were 1.33 ± 0.12 and 6.8 ± 3.5 pmol/g, respectively.

sequences. The targeted database was the sea urchin GLEAN3 gene (protein) predictions database (http://www.hgsc.bcm.tmc.edu/blast.hgsc). The HMM searches were utilized to elucidate ancestral relationships that were not statistically significant in BLAST pairwise sequence similarity searches or evident in the subsequent phylogenetic analysis. HMM searches can be more sensitive, because they utilize a statistical encoding of the consensus sequence for the protein family that captures position-specific conservation information.

Molecular Phylogenetic Analysis. ClustalX2 [Larkin et al., 2007] (http://www.molecularevolution.org/cdc/software/clustalx/), T-coffee [Notredame et al., 2000] (http://www.ebi.ac.uk/Tools/t-coffee/index.html), and MUSCLE [Edgar, 2004] (http://www.drive5.com/muscle/) were used to perform multiple sequence alignments of sea urchin, other invertebrate, and vertebrate receptor sequences. These sequence alignment programs gave similar results for most regions of the proteins. If the alignment was ambiguous, such as in the case of the N- and C-terminal regions of the GPCRs, the region was either ignored or the alignment generated by T-coffee was used. Bootstrapped

(1,000 trials) neighbor-joining trees were calculated using ClustalX2 v2.0.5, and phylogenetic trees determined by bayesian inference were calculated using MrBayes v3.1.2 (http://mrbayes.csit.fsu.edu/). Phylogenetic relationships were mapped with either NJplot (http://pbil.univ-lyon1.fr/software/njplot.html) or TreeView [Page, 1996] v1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Results

Developmental Dynamics of AEA Expression in Early Sea Urchin Embryos

LC/MS was used to quantify AEA in *L. variegatus* sea urchin embryos at the 8–16 cell and mid-blastula 2 stages (fig. 1). The results of these experiments demonstrate the presence of endogenous AEA at these early stages, and

Fig. 2. Dose-response effects of AEA on *L. variegatus* sea urchin embryos. AEA was introduced at the 2-cell stage (2 h post-fertilization) and larvae imaged at the pluteus stage. **A** Untreated control; **B** vehicle control; **C** AEA 5 μM; **D** AEA 10 μM; **E** AEA 20 μM; **F** AEA 25 μM. AEA dose-dependently perturbed development of embryos/larvae compared with controls, preventing them from developing the typical morphology of pluteus larvae, with extended 'arms' (arrows). At the highest doses **E**, **F** cells accumulated in the blastocoel and the ectodermal wall was thickened (**F** inset, arrowhead). Scale bar = 100 μM **A–C**; 50 μM **D–F**. Asterisk = Cells in blastocoel.
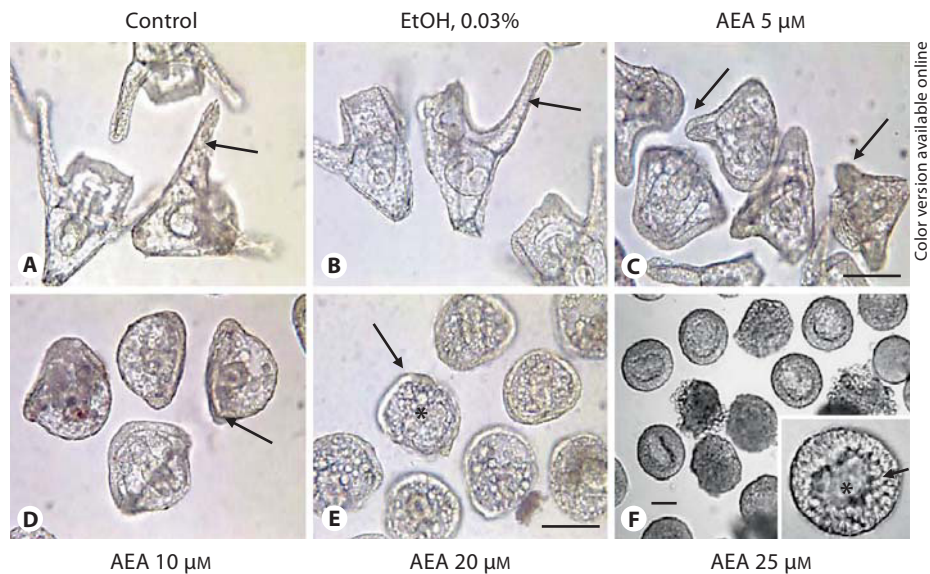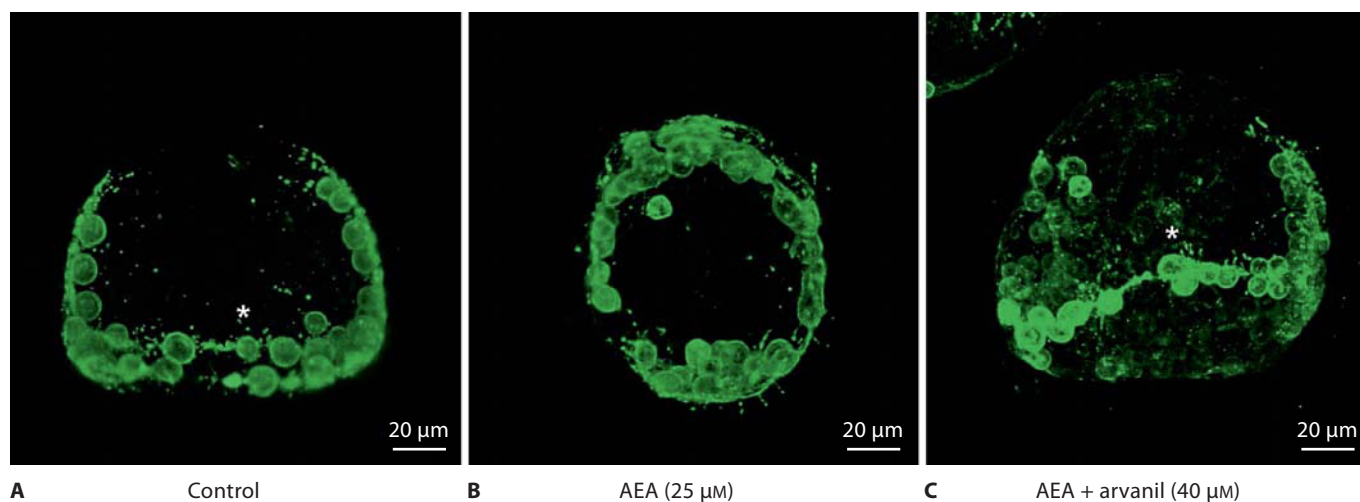

Fig. 3. AEA perturbs the movement of PMC shown in green, during gastrulation. **A** Vehicle control; **B** AEA 25 μM; **C** vanilloid receptor agonist, arvanil 40 μM, partially prevented the effect of AEA, such that the ring of PMC at the vegetal pole is clearly visible in both **A** and **C** (asterisk). Embryos stained with PMC marker 1D5 [Wessel and McClay, 1985].

reveal a clear developmental dynamic, where the amount of AEA at the 8–16 cell stage (~1.33 ± 0.12 pmol/g) increases more than 5-fold to reach ~6.8 ± 3.5 pmol/g by the mid-blastula 2 stage. These findings are consistent with the presence of NAPE-PLD in sea urchin eggs [Bisogno et al., 1997] and preimplantation mouse embryos [Schuel, 2006; Wang et al., 2006c].

AEA as a Teratogen

Results of pharmacologic 'perturb-and-rescue' experiments provide evidence for the teratogenic actions of AEA in early sea urchin development, and demonstrate the ability of a wide spectrum of pharmacologic agents to prevent teratogenesis, including AEA transport inhibitors, and antagonists for CB1, CB2 and TrpV1 (tables 1, 2; fig. 2–4, 7).
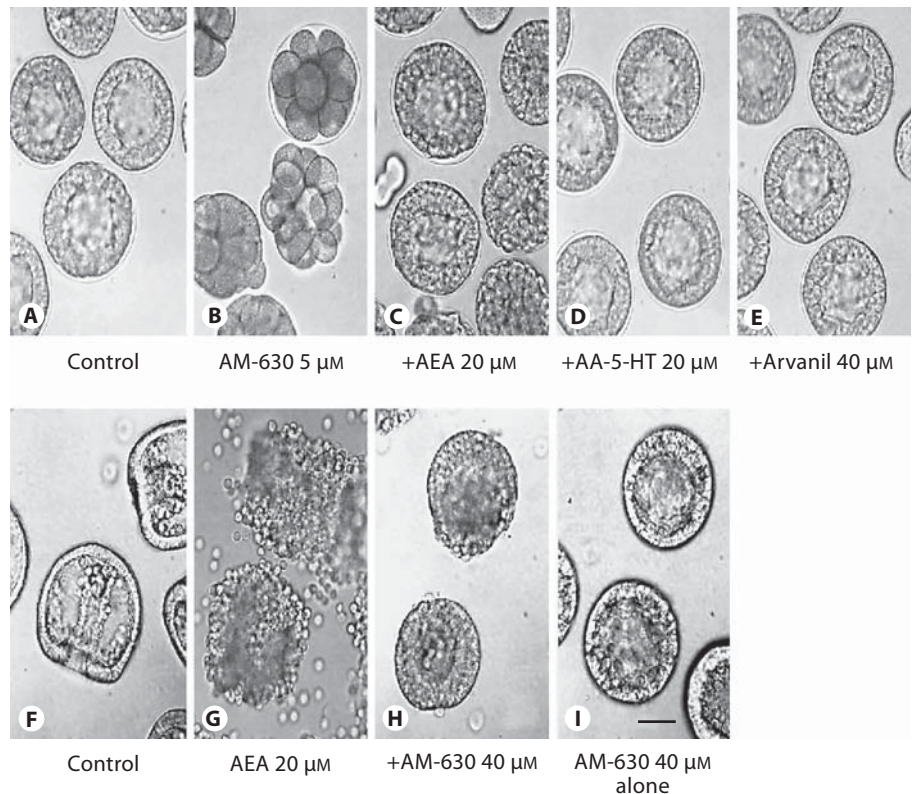
Fig. 4. Developmental dynamics of sensitivity to CB2 receptor antagonist, AM-630 and AEA. Substances were given at the 2–4 cell stage (**B–E**) or at mid-blastula 2 (**G–I**). Controls (**A**, **F**). AM-630 blocked cleavage divisions as shown by embryos composed of multiple blastomeres in **B**. AEA acted as a rescue substance to unblock cleavage divisions, as shown in **C**. However, when given at mid-blastula 2, AM-630 partially rescued from the effects of AEA (**H**). **A–E** Imaged at mid-blastula 2; **F–H** imaged at late gastrula. Scale bar = 50 μm.

Critical Period for AEA Teratogenesis. When introduced at the 2-cell stage, AEA at concentrations as high as 100 μM did not perturb development until after the mid-blastula (pregastrulation) stage. However, after this time, AEA was teratogenic at much lower concentrations, causing malformations to varying degrees, depending on dose (fig. 2). At the highest doses, cells accumulated in the blastocoel and the ectodermal wall was thickened (fig. 2E, F). Although these cells were originally thought to be mesenchymal in origin, immunocytochemistry with antibodies to 1D5, a marker of PMC, revealed that the number of PMC was unchanged by AEA (fig. 3A, B), although their migration pattern was clearly perturbed (fig. 3B). This suggests that the cells in the blastocoel are not PMC, but cells extruded from the ectodermal wall. This view is supported by the observation that, in some embryos exposed to high concentrations of AEA, parts of the ectodermal wall were destroyed and the cells extruded into the culture medium. This is consistent with observations that elevated levels of AEA adversely impact the development of preimplantation mouse embryos [Schuel, 2006; Wang et al., 2006a].

Dose-Dependent Protection from AEA Teratogenesis by Vanilloid Receptor Ligands. Dose-dependent protection from the teratogenic effects of AEA or the VR1/TrpV1 antagonist SB366791 was seen with several different drugs (table 2), including inhibitors of AEA transport (AM-404, VDM-11; partial-complete protection), the CB1 receptor antagonist, AM-251 (fig. 5), the vanilloid receptor (VR1/TrpV1) agonist, arvanil (fig. 4, 5), and the lipophilic 5-HT derivative, AA-5-HT (fig. 4), all three of which exhibited complete protective action.

Teratogenesis and Rescue by CB2 Receptor Ligands. The CB2 receptor antagonist, AM-630, when introduced at the 2-cell stage at doses of 5–10 μM or greater, blocked cleavage divisions followed by death of the embryos (fig. 4B). These effects were partially prevented by AEA, and completely prevented by both AA-5-HT and arvanil (fig. 4D–E), as well as AA-DA (table 2). Interestingly, the action of these drugs as either rescue substance or teratogen was reversed when older embryos (mid-blastula stage) were exposed to the same treatments (fig. 4G–I). Two other non-selective CB receptor agonists, CP55940 and WIN55212-2, when added at the mid-blastula stage, caused an abnormal phenotype similar to AEA, effects that were prevented, to varying degrees, by AM-251, arvanil, and the AEA transport inhibitor, AM-404 (table 2). The teratogenic effects of WIN55212-2 were blocked by

Fig. 5. Action of antagonist of VR1 receptors, SB 366791 and rescue compounds, on development of *S. droebachiensis* embryos. All substances were given at the mid-blastula 2 stage (17 h, 21 min post-fertilization). Specimens were imaged at 26 h, 35 min (**A–F**) or 42 h 05 min (**G–L**) after fertilization. **A**, **G** Control embryos/larvae; **B**, **H** SB366971 1.25 µM (abnormal development); **C**, **I** SB366971 + AA-5-HT 40 µM (normal development); **D**, **J**; **E**, **K** SB366971 + arvanil 40 µM or AM-251 10 µM (good protection); **F**, **L** SB366971 + forskolin 20 µM (moderate protection). Temperature during experiments 14°C. Scale bar = 50 µm (**A–F**) or 100 µm (**G–L**).
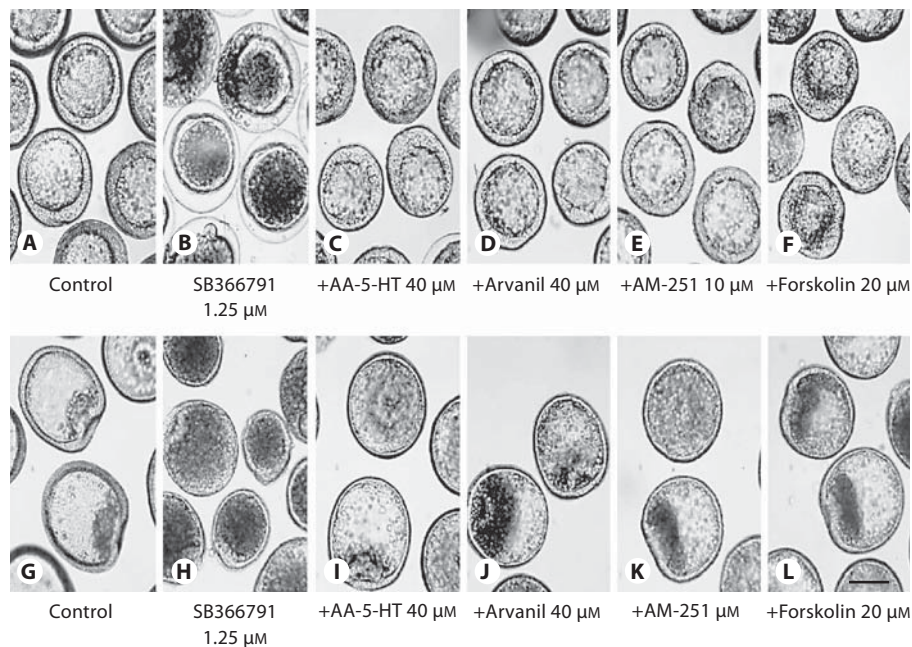

Fig. 6. 'Perturb and rescue' by type 2 cannabinoid receptor ligands. Exposure of embryos at the 2-cell stage to a relatively low dose of the type 2 cannabinoid agonist, WIN552122, causes a severe malformation phenotype, where embryos remain at the blastula stage with dark (likely dead) cells (*) in the blastocoel (**C**). This phenotype can be completely rescued by the type 2 cannabinoid antagonist, SR144528 (**D**). **A** Vehicle control (EtOH, 0.03%); **B** SR144528 (2.5 µM); **C** WIN (5 µM); **D** WIN + SR144528. Scale bar = 20 µm.
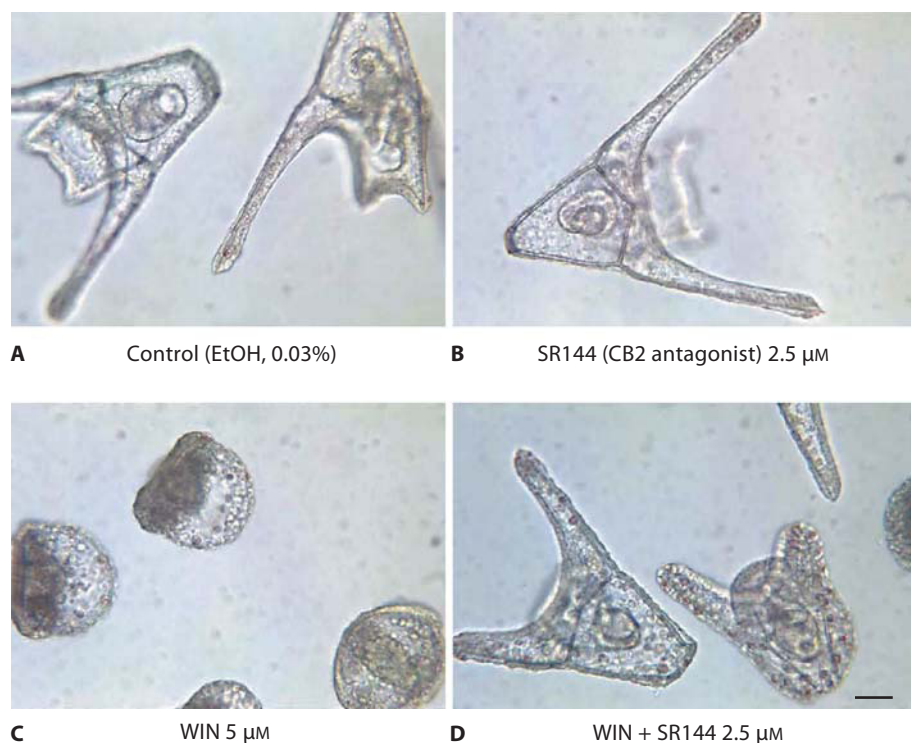
the CB2 antagonist SR144528 (fig. 6). Previous observations showed that THC did not retard the first cleavage division in *S. purpuratus* [Schuel et al., 1987]. Like AEA, WIN55212-2 did not affect cleavage divisions when added at the 2-cell stage. In contrast, CP55940 was a very active teratogen, such that concentrations as low as 1–4 µM caused maximal teratogenic effects from cleavage divisions through the early pluteus stage (fig. 7; table 2), consistent with previous studies on inhibition of the egg jelly-stimulated acrosome reaction in *S. purpuratus* sperm by
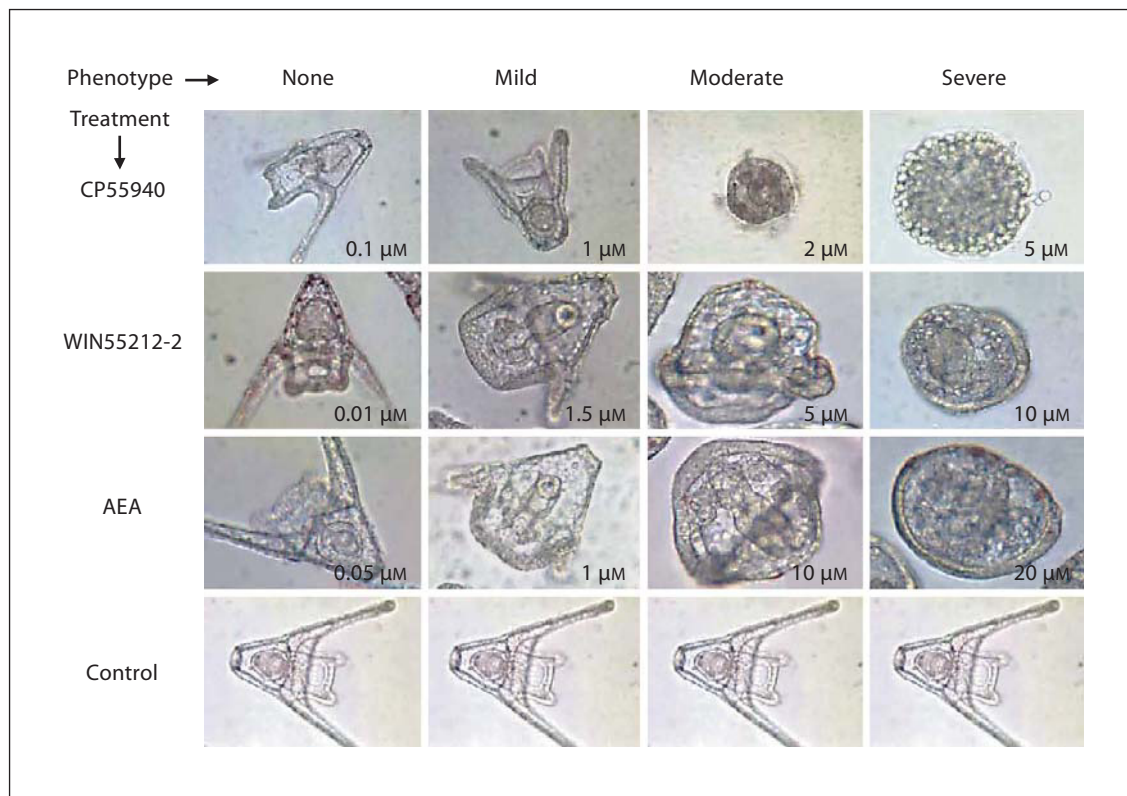
Fig. 7. Dose-dependent malformation phenotypes caused by CB receptor agonists. Dose-dependent malformations caused by different doses of CB receptor agonists, anadamide (AEA), CP55940 (CP), and WIN55212-2 (WIN) given at the 2-cell stage show order of teratogenicity (CP > WIN > AEA) that fits their pharmacologic potency profiles in vertebrates [Breivogel and Childers, 2000; Prather et al., 2000; Palmer et al., 2002].

cannabinergic receptor agonists [Schuel and Burkman, 2005]. Interestingly, the relative teratogenic potency of these drugs (fig. 7) followed the same rank order as their pharmacologic potency in vertebrates [Breivogel and Childers, 2000].

In the sea urchin *S. droebachiensis*, the vanilloid receptor antagonist, SB366791, at concentrations as low as 1–2 μM, caused abnormal post-blastulation development, but did not affect cleavage divisions (fig. 5; table 2). This phenotype was similar to that observed in *L. variegatus* embryos treated with AEA, effects that could be protected against by the same rescue substances. However, unlike AEA-treated embryos, the accumulation of cells in the blastocoel, and extrusion into the culture medium was not followed by destruction of the ectodermal wall, so that abnormal embryos continued to develop, at least to the point when controls reached the late gastrula or prism stage. When these embryos were partially protected by rescue substances, the cells in the blastocoel were not extruded, but accumulated near the blastopore. Another vanilloid receptor antagonist, capsazepine, blocked both cleavage divisions and gastrulation. However, no rescue substances against capsazepine were identified. Even arvanil and resinferatoxin, both very potent vanilloid receptor agonists, did not exhibit protective actions against capsazepine, suggesting that these effects are not mediated by VR/TrpV receptors. Cannabinoid/vanilloid-related ligands also protected embryos/larvae against some non-cannabinoid teratogens, such as the pesticide chlorpyrifos, and several 5-HT$_2$ receptor antagonists (table 2). An especially broad spectrum of protective actions was found for arvanil (table 1).

Receptor Binding
Radioligand-binding assays were performed on untreated embryos and larvae, using the non-selective CB receptor agonist, [^{3}H]CP55940, with or without the selective CB1 antagonist, SR141716 or the selective CB2 an-

Table 3. Binding of non-selective CB receptor ligand, [³H]CP55540, in pluteus, spleen or brain, ± CB2 or CB1 receptor antagonist

Tissue	Total [³H] CP55540 bound fmol/µg (mean ± SEM)	+ CB2 receptor antagonist, SR144	Total [³H]CP55540 bound	+ CB1 receptor antagonist, SR141
Pluteus	0.009 ± 0.002	0.002 ± 0.002	0.007 ± 0.002	0.007 ± 0.005
Brain	0.099 ± 0.039	0.049 ± 0.022	0.160 ± 0.01	0.048 ± 0.012*
Spleen	0.046 ± 0.002	0.006 ± 0.006*	ND	ND

Experiments were run in duplicate with two replicates for each condition.
* p < 0.05 (one-way ANOVA; Newman-Keuls post-hoc test). ND = Not determined.

tagonist, SR144528. These assays produced no evidence of specific binding to CB1, whether they were run on membrane preparations or whole cell lysates. However, when binding assays were run on whole cell lysates, a small amount of [³H]CP55940 binding was seen that could be partially displaced by the CB2 receptor antagonist, SR144528, but not by the CB1 antagonist, SR141716, although the reduction in CP55940 binding by SR144528 did not reach statistical significance (table 3).

Bioinformatics

TrpV and TrpA Phylogenetic Analyses. TrpV and TrpA1 echinoderm homologs were identified consistent with their presence in nematodes, arthropods, and vertebrates. TrpV homologs have 36 ankyrin repeats followed by a cation channel domain, while TrpA1 homologs have 14–17 ankyrin repeats in addition to the cation channel. Alignments containing both TrpA1 and TrpV excluded ~9 of the N-terminal ankyrin repeats of TrpA1 and included only the 6 ankyrin repeats that were closest to the cation channel. Most vertebrate and invertebrate species examined possessed a single TrpA1 ortholog, but multiple TrpV-like homologs (fig. 8A). The vertebrate and invertebrate TrpA1 orthologs formed a monophyletic clade (fig. 8Aa) that also contained a single sea urchin ortholog. Invertebrate TrpV homologs clustered into two main clades – OSM-9 from worm and inactive from fruitfly in the first clade (fig. 8Ab), and the OCR proteins from worm, nanchung from fruitfly and nanLIKE from sea squirt in the second clade (fig. 8Ac). In vertebrates, the TrpV-like homologs also fell into two families, the epithelial calcium channels (TrpV5-6; fig. 8Ad), and the vanilloid receptors (TrpV1-4; fig. 8Ae). Using BLAST searches, we identified 3 sea urchin TrpV-like homologs that clustered with the invertebrate proteins, one with nanchung (fig. 8Ac) and two with inactive (fig. 8Ab). The single *C. intestinalis* (sea squirt) homolog which we included also clustered with the invertebrate nanchung protein (fig. 8Ac).

We tried to elucidate the relationship between the two TrpV-like families in invertebrates, nanchung-like and inactive-like, and the two families in vertebrates, TrpV5-like and TrpV1-like, using profile HMM searches (http://hmmer.janelia.org/), since phylogenetic analyses indicated that the two vertebrate families were more closely related to each other than to either of the invertebrate families. HMM searches can be more sensitive tools for database searches, since searching with a statistically encoded multiple sequence alignment allows position-sensitive conservation information to be utilized in the search. The results from the HMM search using only vertebrate TrpV5-6-like sequences in the query indicated that the nanchung-like GLEAN3 06793 was potentially the sea urchin ortholog of this TrpV subfamily. In this search, the E-value was statistically significant (<1.0⁻³) and the bit score was both positive and >>15 (log₂ of the number of sea urchin sequences in the database), meaning that the sequence was a good match for the profile model [HMMER User's Guide, p. 43]. Interestingly, it was also the closest sea urchin protein to the vertebrate TrpV1-like family, as determined in a second HMM search using only vertebrate TrpV1-4 sequences in the query. Comparing the two HMM searches, it is evident that GLEAN3 06793 was more similar to the profile model of the vertebrate TrpV5-like family than the TrpV1-like family. One potential explanation for these results is that GLEAN3 06793, and the TrpV1-like and TrpV5-like proteins evolved from a common ancestor, but GLEAN3 06793 and the TrpV5-like family members share more sequence similarity and, presumably, have more function in common with the ancestral protein. One implication of this evolutionary hypothesis is that the inactive-like sea urchin proteins might not have direct descendants among the vertebrate TrpV family proteins. A second possibility would be that the TrpV1-like family members evolved from the ancestor to the inactive-like extant sea urchin proteins GLEAN3 21148 and GLEAN3 09599.
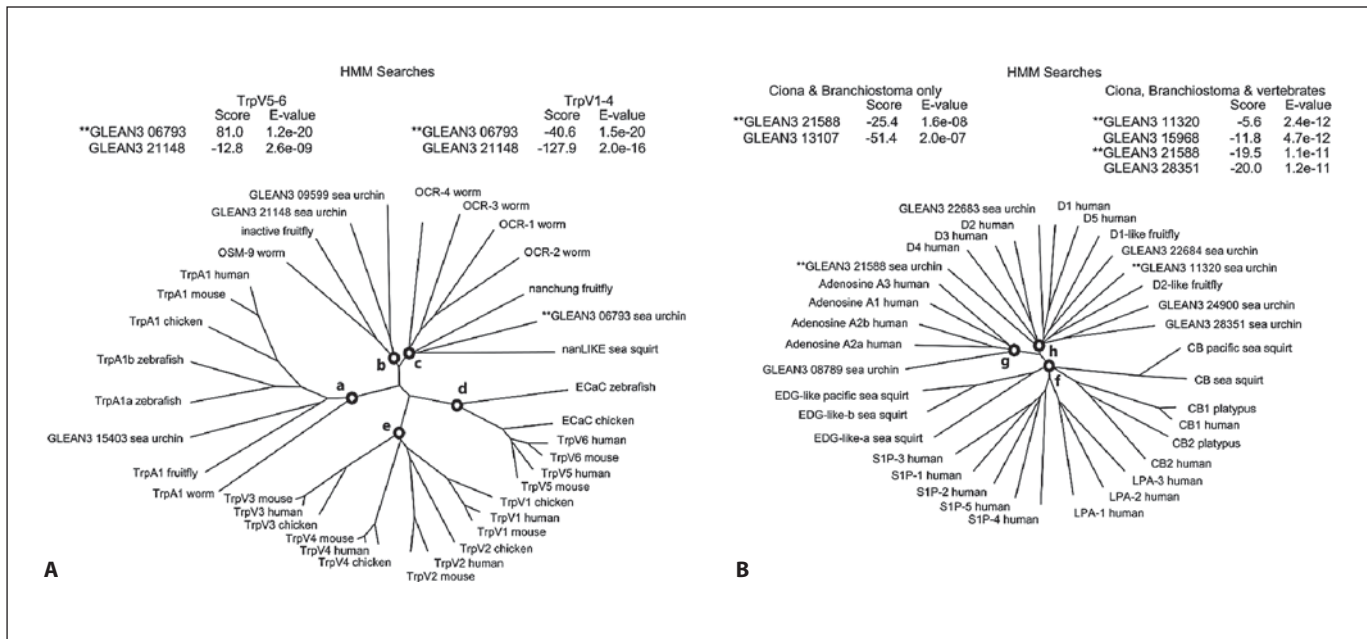
Fig. 8. Phylogenetic relationships of Trp and GPCRs in sea urchins, other invertebrates and vertebrates. **A** Phylogenetic tree of TrpA and TrpV family receptors. Invertebrate and vertebrate species contained a single TrpA gene that formed a monophyletic clade (**a**). Invertebrate TrpV orthologs fell into 2 families, one that contained the worm *(C. elegans)* OSM-9, fruitfly *(D. melanogaster)* inactive (**b**), while the other contained the family of worm OCR proteins, fruitfly nanchung, and sea squirt nanLIKE (**c**). Vertebrate TrpV orthologs also fell into two families, the epithelial calcium channels (ECaC; TrpV5-6) (**d**) and the TrpV1-like channels (TrpV1-4) (**e**). Profile HMM searches against the sea urchin genome, with multiple sequence alignments (MSA) containing vertebrate TrpV family members as the query, indicated that the closest similarity between vertebrate and invertebrate TrpV subunits was between the vertebrate epithelial calcium channels (ECaC; TrpV5-6) (**d**) and the invertebrate sea urchin *(S. purpuratus)* GLEAN3 06793 in the OCR and nanchung clade (**c**). GLEAN3 09599 was also identified as a homolog to the vertebrate TrpV proteins in the profile HMM searches, with E-values of 1.0^{-7} and 1.8^{-3} in the TrpV5-6 and TrpV1-4 searches, respectively. The TrpA1 ortholog in sea urchins, GLEAN3 15403, had E-values higher than 1.5^{-4} in the two HMM searches. **B** Phylogenetic tree of GPCRs. Cannabinoid, Edg-like lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) receptors were found only in vertebrates and the urochordates sea squirt *(C. intestinalis)* and Pacific sea human and fruitfly D_{1-5} dopamine receptors formed a clade with multiple sea urchin GLEAN3 sequences, including the adrenergic-like GLEAN3 21588 and D_1-like GLEAN3 11320 (**h**). HMM searches against the sea urchin genome including all currently identified GPCR receptors, using the urochordate and cephalochordate CB receptors as query, identified the sea urchin GLEAN3 21588 as the most similar. HMM searches with vertebrate CB sequences also included in the search alignment identified the D_1-like GLEAN3 11320 as the most similar. All other GLEAN3 GPCR receptors in the phylogenetic tree had bit scores lower than –55.5 and E-values higher than 3.0^{-7} for the first HMM search containing only the urochordate and cephalochordate sequences. In the search also including vertebrate CB receptors, all other GLEAN3 GPCR receptors in the phylogenetic tree had bit scores lower than –43.1 and E-values higher than 1.5^{-10}.

This hypothesis is supported more by the E-values than by the bit scores from the HMM search with TrpV1-like proteins as the query.

Cannabinoid GPCR Phylogenetic Analysis. Consistent with published reports of the earliest GPCR cannabinoid receptor being identified in the urochordate *C. intestinalis* [Elphick et al., 2003; Matias et al., 2005] and cephalochordate *B. floridae* [Elphick, 2007], and reports of the lack of cannabinoid receptors in the sea urchin genome [Burke et al., 2006; Sodergren et al., 2006], we did not identify any clear echinoderm GPCR cannabinoid receptor orthologs using pairwise sequence similarity searches. BLAST searches with human CB1 or CB2 proteins as query sequences identified sea urchin proteins with top hits on the dopamine D_1 (GLEAN3 22683, GLEAN3 28351), and related receptors [5-HT$_4$ (GLEAN3 08947), H$_2$ (GLEAN3 15968), α_1-adrenergic (GLEAN3 14555 and GLEAN3 20361)] when blasted back against the human genome (data not shown). In addition, different results were obtained when blasting the sea urchin genome depending on whether the query was the human CB1 (GLEAN3 08947, GLEAN3 15968, GLEAN3 14555,

GLEAN3 20361 as top hits) or the human CB2 receptor sequence (GLEAN3 28351 as the top hit).

A phylogenetic analysis of representative cannabinoid-related and other GPCRs yielded a tree (fig. 8B) with three monophyletic clades. One clade contained the vertebrate CB, Edg-like lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) receptors, as well as EDG-like and CB GPCRs from the urochordates *C. intestinalis* (sea squirt) and *C. savignyi* (Pacific sea squirt) (fig. 8Bf). No other invertebrate sequences, except for the invertebrate chordates, clustered within this group. Human A1, A2a, 2b, and A3 adenosine receptors and a single sea urchin adenosine receptor formed a separate monophyletic clade (fig. 8Bg). In contrast, human D_{1-5} and fruitfly dopamine receptors formed a clade with multiple sea urchin GLEAN3 sequences, including GLEAN3 21588 and GLEAN3 11320 (fig. 8Bh).

We also used HMM searches to query the sea urchin genome for GPCR. Given that a single GPCR cannabinoid receptor had been identified in *C. intestinalis* and *B. floridae*, a receptor proposed to be the common ancestor of both vertebrate CB1 and CB2 receptors [Elphick et al., 2003; Elphick, 2007], and the fact that the ascidian and mammalian CB receptors shared only 29% sequence identity suggesting significant functional divergence in the cannabinoid receptor after branching of urochordates and cephalocordates from the vertebrate lineage, we decided to use the urochordates *C. intestinalis* (sea squirt) and *C. savignyi* (Pacific sea squirt) and cephalochordate *B. floridae* CB receptors to query the sea urchin genome with the HMM search strategy, since the single CB receptor in urochordates and cephalochordates shared the nearest ancestry to the pre-gene duplication echinoderm homolog that gave rise to the vertebrate CB receptor and thus was the best candidate to clarify the evolutionary origin of the GPCR cannabinoid receptor. Our first query MSA consisted of the two urochordate and the cephalochordate CB receptor sequences. The top hit in this HMM search was the sea urchin GLEAN3 21588, an apparent adrenergic ortholog based on NCBI annotations, and the second hit was an H_2-like sea urchin protein (GLEAN3 13107), with bit scores of –25.4 and 51.4 and E-values of 1.6^{-8} and 2.0^{-7}, respectively. Therefore, GLEAN3 21588 is potentially derived from an ancestor common to both the urochordate/cephalochordate and vertebrate CB receptors. If several vertebrate CB1 and CB2 receptors were included with the *Ciona* and *Branchiostoma* CB sequences in the query MSA, the top 4 hits included GLEAN3 21588 and 3 other sea urchin proteins from the dopamine/histamine family [GLEAN3 11320

(D_1-like), GLEAN3 15968 (H_2-like) and GLEAN3 28351 (D_1-like)] with bit scores ranging from –5.6 to –20.0 and E-values ranging from 2.4^{-12} to 1.2^{-11}. These HMM search results against the sea urchin genome using the CB receptors from urochordates, cephalochordates and/or vertebrates as query suggest that either the sea urchin *(S. purpuratus)* GLEAN3 21588, an adrenergic ortholog, or the D_1-like ortholog (GLEAN3 11320) were the most similar GPCRs. This result also suggests that these GPCRs are potentially derived from an ancestor common to both the urochordate/cephalochordate and vertebrate CB receptors. Given the variations in outcome of the profile HMM searches, pharmacologic responses will be needed to further identify the receptors acting as the source of the endocannabinoid response in sea urchin embryos.

Discussion

Developmental Dynamics of AEA in Early Echinoderm Embryos

AEA was detected in 8–16 cell embryos by LC/MS (fig. 1), where levels of ~1.33 + 0.12 pmol/g were measured. By the mid-blastula 2 stage, AEA levels had increased more than 5-fold to ~6.8 + 3.5 pmol/g. As discussed below, 2-cell (cleavage stage) embryos were insensitive to any dose of exogenous AEA. The reasons for this are unclear, since 2-cell embryos were not analyzed by LC/MS. It is possible that very low levels of AEA are present at this early stage, whereas the relevant receptors have not yet been expressed. Nevertheless, the current data do show detectable levels of AEA as early as the 8–16 cell stage, which have increased significantly by the mid-blastula stage. Such developmental dynamics may drive critical periods of sensitivity to the teratogenic effects of AEA and the other drugs tested, similar to the deleterious effects of inappropriately high levels of AEA on early vertebrate embryos [Wang et al., 2006a; Turco et al., 2008; Wang et al., 2008]. Indeed, the adverse effects of exogenous AEA, cannabinergic receptor agonists, and AEA transport inhibitors suggest the existence of an endogenous AEA tone in developing sea urchin embryos similar to the regulatory system that operates in early mouse embryos [Schuel, 2006; Wang et al., 2006a; Wang et al., 2006b], zebrafish and frogs [Migliarini et al., 2006; Migliarini and Carnevali, 2008, Migliarini and Carnevali, 2009]. Together, these findings suggest that AEA-mediated regulation of early embryonic development had an ancient origin in the evolutionary history of the endocannabinoid system.

Stage-Specific Sensitivity to AEA and Receptor Ligands

Although cleavage stage embryos were insensitive to the teratogenic effects of AEA (table 2), AEA clearly protected embryos from the teratogenic effects of the CB2 receptor antagonist, AM630 (fig. 4), suggesting that receptors mediating effects of these ligands allow them to exert their typical pharmacologic effects. Sensitivity to the teratogenic effects of AEA only emerged after transition of embryos to the blastula stage, when moderate doses (5–10 μM) caused significant malformations, which increased in severity with dose (fig. 2). Higher doses of AEA (15–25 μM) disrupted movement of cells along the inner ectodermal surface, and disturbed early patterning of PMC (fig. 3). However, these doses did not appear to decrease the number of PMC, but rather caused accumulation of ectodermal cells in the blastocoel. At the highest doses, these cells were sometimes released into the culture medium, suggesting toxicity.

There is an obvious inconsistency between the results of our 'perturb-and-rescue' experiments using CB receptor ligands and failure to detect CB1 and CB2 orthologs in the sea urchin genome, despite evidence from radioligand-binding studies in sea urchin sperm suggesting the presence of CB receptors similar to those in mammalian brain and peripheral organs [Chang et al., 1993], where AEA, THC and other CB receptor agonists elicit biological responses similar to those observed in mammalian neurons and somatic cells [Schuel et al., 1994; Berdyshev, 2000; Schuel and Burkman, 2005]. However, we believe that our in silico evidence, discussed below, provides clues to what receptors may be mediating these 'perturb-and-rescue' effects and the observed developmental regulation by AEA and other endocannabinoids in sea urchins.

Involvement of Trp and GPCRs in Developmental Endocannabinoid Signaling

The partial protective actions of the vanilloid receptor (VR1/TrpV1) agonist arvanil against the teratogenic effects of AEA (fig. 3), together with the ability of cannabinoid receptor ligands like AM-251 and AM-630 to protect against the teratogenic effects of the TrpV1 antagonist, SB366971 (fig. 4, 5; table 2), suggest that although AEA may act via TrpV1 or TrpA1 receptors, as in vertebrates [Al-Hayani et al., 2001], it may also utilize GPCRs to exert its developmental effects. Lipophilic arachodonyl 5-HT and DA (AA-5-HT, AA-DA) showed good protective actions against the teratogenic effects of AEA, as well as CP55940 (a direct derivative of THC), and SB366971 (tables 1, 2). Although these actions are likely mediated

by VR1/TrpV1 receptors, which mediate actions of these drugs in vertebrate brain [Marinelli et al., 2007; de Novellis et al., 2008], they might also exert protective actions via ligand-gated ion channels and GPCR-like 5-HT$_{3A}$, 5-HT$_{1A}$ or D$_2$-like receptors, which are known to bind AEA in vertebrates [Barann et al., 2002; Centonze et al., 2004; Przegalinski et al., 2005; Russo et al., 2005]. Alternatively, AA-5-HT and AA-DA might interact with heterodimeric receptors like D$_1$/D$_2$ [So et al., 2007; Faron-Gorecka et al., 2008] or mosaic receptors like CB1/D$_1$/A2A or CB1/D$_2$/A2A [Fuxe et al., 2008; Marcellino et al., 2008; Navarro et al., 2008; Ferré et al., 2009], where the CB component is replaced by another GPCR or is not required for formation of the mosaic receptor. Although heterodimer/mosaic receptors have not yet been reported in invertebrates, the possibility of their existence is worth considering. Such a scenario could help explain the finding that, even without CB receptors, CB receptor ligands like CP55940 (a non-classical cannabinoid), WIN55212-2, and AEA exhibit similar dose-dependent teratogenic activities in developing sea urchins (fig. 7) as their potency profiles in vertebrate tissues would predict [Breivogel and Childers, 2000; Prather et al., 2000; Palmer et al., 2002].

Phylogeny of the Endocannabinoid System

The endocannabinoid system in mammals consists of a family of lipid-signal molecules that are endogenous ligands for cannabinoid receptors, and enzymes responsible for their synthesis and degradation [Mechoulam et al., 2006; Sugiura et al., 2006]. *N*-Archidonoyl ethanoloamine (AEA, anandamide) was the first endogenous ligand for cannabinoid receptors to be discovered [Di Marzo et al., 1999]. Currently known endocannabinoids also include ethanolamides, sn-2 arachidonyl glycerol (2-AG), and virodhamine. Since no cannabinoid GPCR orthologs have been identified in the sea urchin genome, it is possible that cannabinoid receptors evolved after appearance of the endocannabinoid ligands [McPartland et al., 2006; McPartland et al., 2007a], following separation of echinoderms from the extant vertebrate lineage. This view is consistent with evidence for a primitive cannabinoid-like receptor in the urochordate, *C. intestinalis* [Elphick et al., 2003; Matias et al., 2005] and cephalochordate, *B. floridae* [Elphick, 2007]. The need, then, is to identify the extant sea urchin receptors that are candidates for a cannabinoid-responsive receptor system in the absence of CB receptor orthologs.

Potential receptors that are present in the sea urchin genome that might mediate the developmental effects of AEA and other endocannabinoids include a number of

ankyrin repeat-containing transient receptor potential (Trp) cation channels, annotated as TrpA1 or TrpV1. Both bind THC and AEA in vertebrates [Jordt et al., 2004; Kim et al., 2007; De Petrocellis et al., 2008], and vertebrate TrpA1 has recently been shown to respond to multiple phytocannabinoids, including THC [De Petrocellis et al., 2008]. However, the results of the present study suggest that GPCRs may also be involved in endocannabinoid actions in developing sea urchins. These receptors might be in the form of heterodimers or mosaic receptors.

Based on bioinformatics analyses (fig. 8A; online suppl. fig. S1), sea urchin Trp receptor homologs exist for both the TrpA1 family (vertebrates and invertebrates) and TrpV1-6 family (vertebrates), but no CB-like receptors were found, consistent with the lack of cannabinoid receptor orthologs in the sea urchin genome [Burke et al., 2006; Sodergren et al., 2006]. One likely candidate for an endocannabinoid-responsive GPCR would be the ancestral receptor from which the CB receptor evolved. We identified a sea urchin dopamine-like homolog as potentially being the nearest match to this CB receptor, using profile HMM searches (fig. 8B; online suppl. fig. S2). This result is interesting for several reasons. First, the vertebrate D_2 receptor has been reported to bind endocannabinoids [Giuffrida et al., 1999; Centonze et al., 2004], raising the possibility that (1) the sea urchin dopamine receptor homolog may possess an endocannabinoid response and (2) endocannabinoid binding may have been a primordial function of the common GPCR ancestor that gave rise to the vertebrate CB receptors. Second, the presence of CB1-D_2 heterodimers [Navarro et al., 2004; Ferré et al., 2008; Marcellino et al., 2008] along with the formation of D_1-D_2 heterodimers [So et al., 2007; Faron-Gorecka et al., 2008] have been reported in vertebrate tissues. Third, Beggs et al. [2005] have found D_2-like receptors in the arthropod *Apis mellifera* (honey bee) that overlap in expression with D_1-like receptors, suggesting that this may reflect the presence of D_1-D_2 receptor complexes in some cells. Given the extent of reported interactions between these GPCRs in organisms ranging from arthropods to vertebrates, it is possible that endocannabinoid-dopamine receptor interactions may have been a primordial function that was conserved after the gene duplication that resulted in the CB receptor.

In addition, we cannot rule out the use of other noncanonical receptors by AEA and other cannabinoid receptor ligands. In this context, it is worth noting evidence for cannabinoid receptors in vertebrates in addition to CB1 and CB2, such as the orphan receptor GPR55 [McPartland et al., 2007a; McPartland et al., 2007b], which has been identified as a novel cannabinoid receptor with ligand-binding and signaling properties partly similar to and partly unique from CB1 and CB2 [Pertwee, 2007; Ryberg et al., 2007; Lauckner et al., 2008]. Interestingly, AM-251, an analog of the CB1-selective antagonist SR141716 [Palmer et al., 2002], is a potent agonist for human GPR55 [Ryberg et al., 2005; Ryberg et al., 2007] and functions as a CB receptor agonist, mimicking the inhibitory effects of AEA and THC on fertility of sperm in the sea urchin *S. intermedius* [Berdyshev, 1999]. BLAST searches using human GPR55 (Q9Y2T6) as a query did not detect an obvious ortholog in the NCBI *S. purpuratus* genome, instead hitting on XP_786638, a type 2 somatostatin receptor, originally annotated as CB1-like. Future studies will be required to determine whether any of the candidate receptors identified here actually function as receptors for endocannabinoids or cannabinoid receptor ligands in developing sea urchins and sperm.

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